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Identification of a New Control Region in the Genome of the DDP Strain of BK Virus Isolated From PBMC

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The various strains of human polyomavirus BK (BKV) show a marked heterogeneity in the noncoding control region (NCCR), which includes the origin of replication and the regulatory region for early and late transcription. A new BKV strain (DDP, U91605) was identified by direct detection and sequencing of PCR products of BKV-NCCR DNA obtained from PBMC samples of HIV-positive or -negative subjects. The DDP strain NCCR sequence showed an organisation not described previously in vivo with the maximum homology with the archetypal strain (WW) (M34048), as compared with those collected in GenBank. Structurally, P_{68}, Q_{39}, and S_{68} boxes were perfectly conserved, whereas the R_{63} box was completely deleted. This deletion involves the loss of sequences able to bind cellular factors essential for the DNA transcription, such as NF1 binding sites, normally present twice in the R box and the modification of SP1. It is possible that these rearrangements represent a cause of the loss of the VP1 region observed in 9/22 PBMC samples and never observed in urine isolates, which are similar to the WW strain. ***J. Med. Virol. 58:413–419, 1999.***

KEY WORDS: human polyomavirus; BKV; DDP strain; NCCR; transcriptional factors; binding sites

INTRODUCTION

The original strain of BKV was first isolated by Gardner et al. (1971) from the urine of a kidney transplant patient and has been used commonly for experimental studies together with the closely related strain BKV (Dunlop) [Seif et al., 1979]. A number of strains has been isolated successively [Yoshiike and Takemoto, 1986]. These strains have generally been subjected to repeated cell culture passages before detailed characterisation was initiated and probably the passages led to rearrangements and deletions of sequences involved in control functions of the DNA transcription [Watanabe et al., 1984; Rubinstein et al., 1987; Jin and Gibson, 1996]. In one case, the viral DNA was cloned directly from several litres of urine collected from the same patient and this strain was proposed as the archetypal strain of BKV (WW), [Mew et al., 1981; Chauhan et al., 1984; Rubinstein et al., 1987].

DNA sequencing of the BKV strains has revealed a striking sequence conservation in the polypeptide-encoding regions of the genome [Seif et al., 1979; Tavis et al., 1989]. In contrast, a high degree of heterogeneity has been demonstrated in the approximately 400 base pair (bp) long noncoding control region (NCCR), which includes the origin of replication and the regulatory region for early and late transcription [Yoshiike and Takemoto, 1986]. In order to visualize the rearrangements found in different NCCR variants, the transcriptional control region of the proposed archetypal BKV strain (WW) has been divided arbitrarily into three sequence blocks, called P (68 bp), Q (39 bp), and R (63 bp) and a late leader region S (63 bp) [Markowitz et al., 1988]. Most BKV isolates reveal a NCCR with considerable variations due to deletions, duplications, and rearrangements of these blocks [Markowitz and Dynan, 1988; Moens et al., 1995].

NCCR variations can be detected both in naturally occurring variants of BKV and JCV (the other human polyomavirus), isolated and sequenced directly from human samples [Azzi et al., 1996], as well as in BKV

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strains obtained after several passages in cell culture, a process predisposing for NCCR rearrangements [Jin and Gibson, 1996].

The analysis of sequences of the NCCR blocks has shown the presence of different binding motifs for cellular transcription factors such as the consensus motifs for CRE (P block), Sp-1 (Q and R blocks), and NF-1 (P, Q, R, and S blocks) [Johnsen et al., 1995; Moens et al., 1995]. Mutations or deletions at these sites can affect *in vitro* replication, the host cell permissivity the promoter/enhancer or the transforming activities *in vitro* [Yoshiike and Takemoto, 1986; Sundsfjord et al., 1990; Moens et al., 1995].

Previous work in our laboratory made possible the detection of BKV and JCV from peripheral blood mononuclear cells (PBMC) of HIV-positive or negative patients attending a Centre for Sexually Transmitted Diseases (STD) by means of PCR, using different sets of primers specific for the NCCR or for the VP1 capsid protein coding region. As far as the JCV genome was concerned, the VP1 and the NCCR sequences were always present concomitantly in the same samples, whereas the BKV-VP1 sequence was detected only in 50% of the samples positive for the NCCR [Degener et al., 1997].

To clarify the role of this virus in human pathology, it is therefore important to know in detail the NCCR sequence of BKV, isolated directly from biological samples. For this purpose, the NCCR of BKV isolates was examined both from urine and PBMC obtained from subjects positive for BKV DNA included in the previous study. Passages in culture were avoided and the NCCR was amplified by PCR directly from the specimens. PCR products were subsequently sequenced. A novel structure present in the BKV genome with the characteristic features of a control region was identified in PBMC samples. A detailed nucleotide analysis of this structure is described highlighting its possible relationship to the conserved or lost binding sites for cellular transcriptional factors.

MATERIALS AND METHODS

Specimens and Treatment

Thirteen urine specimens and 22 peripheral blood mononuclear cells (PBMC) were collected from HIV-positive (13) and HIV-negative (9) patients, attending a Centre for Sexually Transmitted Diseases (STD) of the San Gallicano Institute of Rome (IRCCS).

One milliliter of urine was centrifuged for 2 minutes. Sediments were washed with PBS and resuspended in 100 μl of distilled water to lyse the cells. After heating at 95°C for 5 minutes and spinning in a microcentrifuge for 10 seconds, the supernatants were collected and stored at –20°C until PCR amplification [Jin et al., 1995]. PBMC were isolated from whole heparinised blood samples by gradient centrifugation [Boyum, 1968], washed twice in PBS and 10 cell aliquots were stored at –20°C.

All the samples were submitted to a rapid DNA extraction by QIAamp Tissue Kit (Qiagen Inc., M-Medical-Genenco). DNA yield was determined by measuring its concentration in the eluate by absorbance at 260 nm and then 1 μg of total DNA was used directly for PCR amplification.

Condition for PCR

DNA samples suitability for PCR was first checked by amplification of the HLA gene using primers GH26 and GH27 for the HLA Dq Alpha locus (Synthetic Genetics, San Diego, CA).

Only positive samples were submitted to further amplification of the target DNA sequences. PCR amplifications were carried out in a GeneAmp PCR System 2400 (Perkin-Elmer Cetus, Emeryville, CA). All experiments were done in parallel with positive and negative controls [Kwok and Higuchi, 1989].

PCR for NCCR of BKV Genome

A nested PCR was carried out using two pairs of primers (BKTT1 and BKTT2) annealing to invariant regions flanking the BKV NCCR. After 40 amplification cycles BKTT1 (positive sense) and BKTT2 (negative sense) primers were expected to generate a 748 bp DNA fragment [Flaegstad et al., 1991].

The second pair of primers [BK1 (positive sense) and BK2 (negative sense)] was designed to amplify a portion of the first round PCR product. The samples were subjected to 29 amplification cycles and the expected BKV product was 354 bp in length [Markowitz et al., 1993]. The PCR products were detected by ethidium bromide staining after electrophoresis on 3% agarose gel.

PCR for VP1 Region of BKV Genome

PCR primers were chosen to anneal to the region flanking the VP1 subtype-specific region of BK virus [Jin et al., 1995]. Primers VP1-7 (positive sense), 5′ATCAAAGAACTGCTCCTCAAT-3′ (nucleotides 1480–1500) and VP1-2R (negative sense), 5′-GCACTCCCTGCATTTCCAAGGG-3′ (nucleotides 2038–2059) (numbers of BKV from Seif et al., 1979) were expected to generate a 579-bp fragment for BKV after 35 amplification cycles in the first round PCR. Reactions were subjected to 2 minutes of denaturation at 94°C, followed by 35 rounds of an amplification cycle consisting of 1 minute at 91°C, 1 minute at 55°C and 1 minute at 72°C, followed by one extension cycle consisting of 4 minutes at 74°C [Pietropaolo et al., 1998].

The second pair of primers [327-1 (positive sense) and 327-2 (negative sense)] was designed to amplify a portion of the first round PCR product. The samples were subjected to 35 amplification cycles and the expected BKV product was 327 bp in length [Jin et al., 1995].

The PCR products were detected by ethidium bromide staining after electrophoresis on 3% agarose gel.

Sensitivity of the PCR for the BKV NCCR and VP1

The sensitivity of the two nested PCR assays for the NCCR and for the VP1 of BKV was estimated by am-

TABLE I. Detection of the BKV VP1 Region by PCR in NCCR Positive Samples

Samples	No. subjects	VP1-PCR analysis	
		Positive	Negative
PBMC	22	9	13
Urine	4	4	0

plification of serial dilutions of infected tissue culture fluids containing BK virus. Both PCR assays reached the same dilution points [Pietropaolo et al., 1998].

Sequencing of BKV NCCR and VP1

PCR products corresponding to the NCCR and to the VP1 region were purified before sequencing to remove the excess of primers with QIAquick PCR purification kit according to QIAGEN protocol. DNA sequencing was performed by automatic DNA sequencer (Applied Biosystem, Perkin Elmer, mod. 370 A) according to the specifications of the manufacturer (Amplicycle kit, Perkin-Elmer). In this procedure 300–500 ng of BKV-DNA and 0.8 pmol of primer was used in each sequencing reaction. Primers BK1 and BK2 were used for sequencing the NCCR and 327-1 and 327-2R for sequencing VP1 region.

Sequence Data Analysis

Sequence data were organised and analysed by using the Genetics Computer Group sequence analysis software package [Devereux et al., 1984] on a VAX computer.

Restriction Fragment Length Polymorphism Assay

In order to subtype BKV, the PCR products were subjected to restriction fragment length polymorphism (RFLP) assay [Jin et al., 1995]. Ten microliters of PCR product was digested with 1–2 units of appropriate endonucleases in a total of 20 μl of the supplied buffer at 37°C for 2 hours. A two-step approach was performed: digestion with Alu I enabled to distinguish subtypes I and II of BKV from subtypes III and IV; subsequently, the digestion patterns produced by Xmn I and Ava II, respectively, were used to distinguish strains of subtype I from II and subtype III from IV. The reaction mixture was submitted to electrophoresis on an ethidium bromide-stained 3% agarose gel.

RESULTS

This study examined the data obtained in 22 PBMC belonging to 13 HIV-positive and 9 HIV-negative subjects. All PBMC samples were positive for BKV NCCR, as revealed by specific PCR (Table I). An additional specific nested PCR was undertaken to detect the presence of VP1 region that was revealed in 6 (46%) of the 13 PBMC samples from HIV-positive subjects, and in 3 (33%) of the 9 PBMC specimens from HIV-negative subjects (no significant difference, $P = .41$).

Thirteen urine specimens were also analysed: 4 were found positive both for VP1 and NCCR that were concomitantly present in all samples (Table I). The analysis of the urinary and PBMC VP1-PCR products was carried out by RFLP assay and showed that they all belonged to the subtype I BKV, as confirmed by sequence analysis conducted with an automatic DNA sequencer (data not shown).

All 22 PBMC samples and 4 urine specimens were also sequenced for NCCR. The sequences of the BKV NCCR from PBMC of all patients showed an identical structural organisation and relevant differences were found when these were compared with those available in the GenBank. Consequently, a novel "consensus" sequence of BKV NCCR (strain DDP) was constructed and registered with access number U91605 in EMBL (Fig. 1).

The structural analysis of DDP strain sequence showed the maximum homology with the archetypal strain WW since P, Q, and S boxes were perfectly conserved, whereas the R box was completely deleted. Figure 1 shows a comparison between the NCCR structures reported for different strains selected for homology with DDP strain.

When urine specimens were analysed, NCCR amplification products were sequenced and results of comparative analysis revealed a structure like the archetypal BKV control region (WW) with the presence of P, Q, R, and S blocks (Fig. 1).

Samples recovered from the same patients but from different cell types (PBMC and epithelial urinary tract cells) did not show the same organisation and structure of the NCCR. Figure 2 shows some representative sequences regarding BKV NCCRs from different PBMC samples. Only a few point mutations were found in the various sequences (C 113→T, C 119→T, G 185→A, C 217→G, A 225→T, C 278→T) with no differences in the nucleotide number of the amplified fragments. Figure 3, finally, shows the main binding sites for cellular transcriptional factors present in the DDP strain as compared to the WW strain. The deletion of R-box causes the loss of two NF-1 sites and the 2 nucleotide shortening of SP-1 binding site. Moreover, other binding sites, located in boxes P and Q, were conserved but the distance between these sites and the ATG initiation codon for late transcription was modified.

DISCUSSION

BKV DNA was analysed by PCR in PBMC and in urine samples collected from HIV-positive and negative patients. The analysis of sequences obtained from NCCR amplification products from different subjects, compared with those available in GenBank, made it possible to design a novel "consensus" sequence of a BKV-DDP strain obtained from PBMC of both HIV-positive and HIV-negative subjects. This sequence has been sent and registered under access number U91605 in EMBL.

The DDP strain NCCR sequence showed the highest homology with the TU strain (M34049) and with the

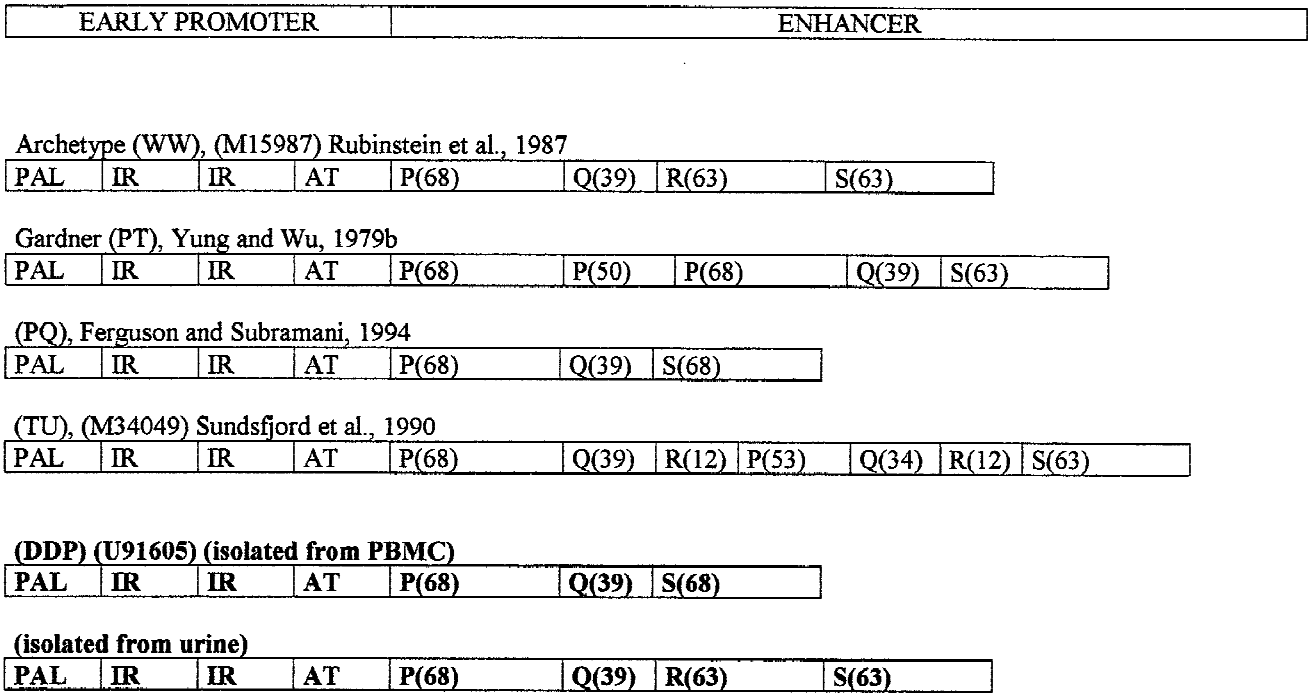

Fig. 1. Structures of the noncoding control regions of various BKV strains. The early promoter, which is the same in all strains consists of a palindrome (PAL), two inverted repeats (IR), and an AT-rich region (AT) of 20bp. The archetype strain WW has the enhancer structure that contains single P (68bp), Q (39bp), R (63bp), and S (63bp) blocks. Homologous sequences are aligned vertically and shaded similarly. BKV sequences isolated in our laboratory from PBMC and urine samples are indicated in bold. Numbers shown are sequence lengths in base pair. The GenBank accession number of the sequences are shown in the brackets.

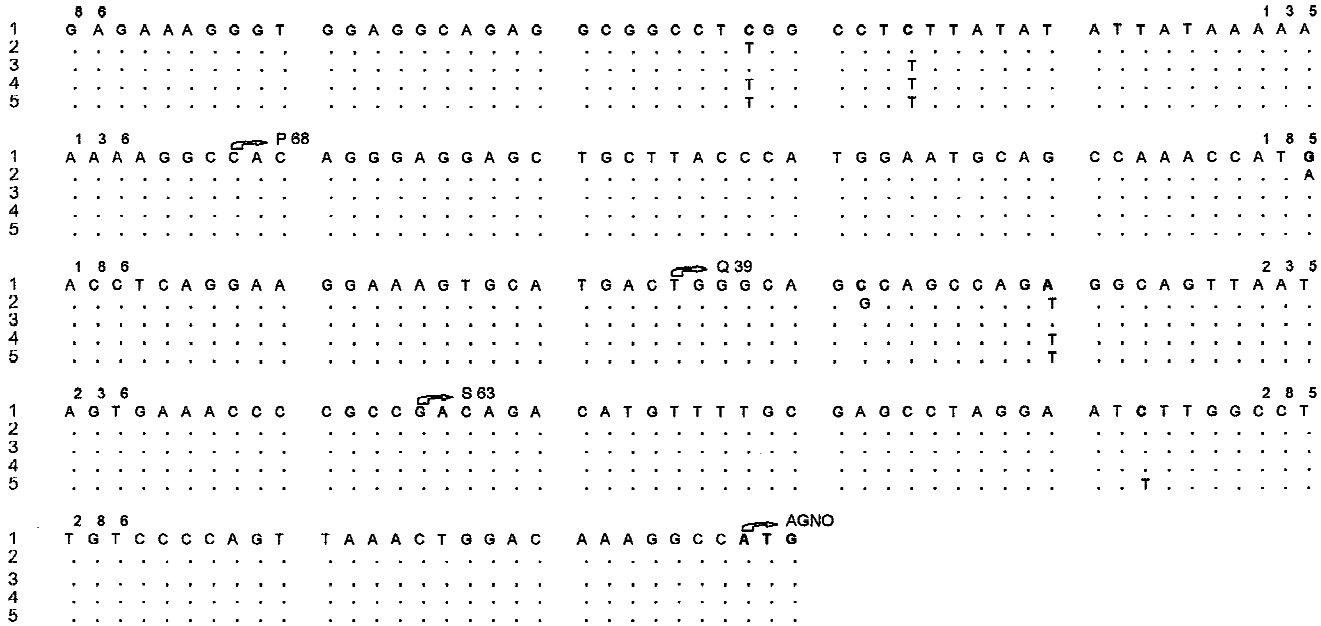

Fig. 2. Nucleotide sequences of the BKV NCCR determined directly from different PBMC samples. Point mutations among sequences are indicated. Regulatory boxes (P, Q, and S) and the ATG initiation codon for the agnoprotein (AGNO) are shown. The numbering system is that used by Seif et al. [1979].

archetypal strain (WW) (M34048) as far as the P_{68} and Q_{39} boxes are concerned, but was characterised by a constant deletion of the R_{63} box. It had been postulated that the natural archetypal NCCR of BKV consists of a linear array of functional DNA elements, and that DNA rearrangements of this region observed in laboratory strains are generated during the propagation of viral isolates in cell cultures [Negrini et al., 1991; Mo-

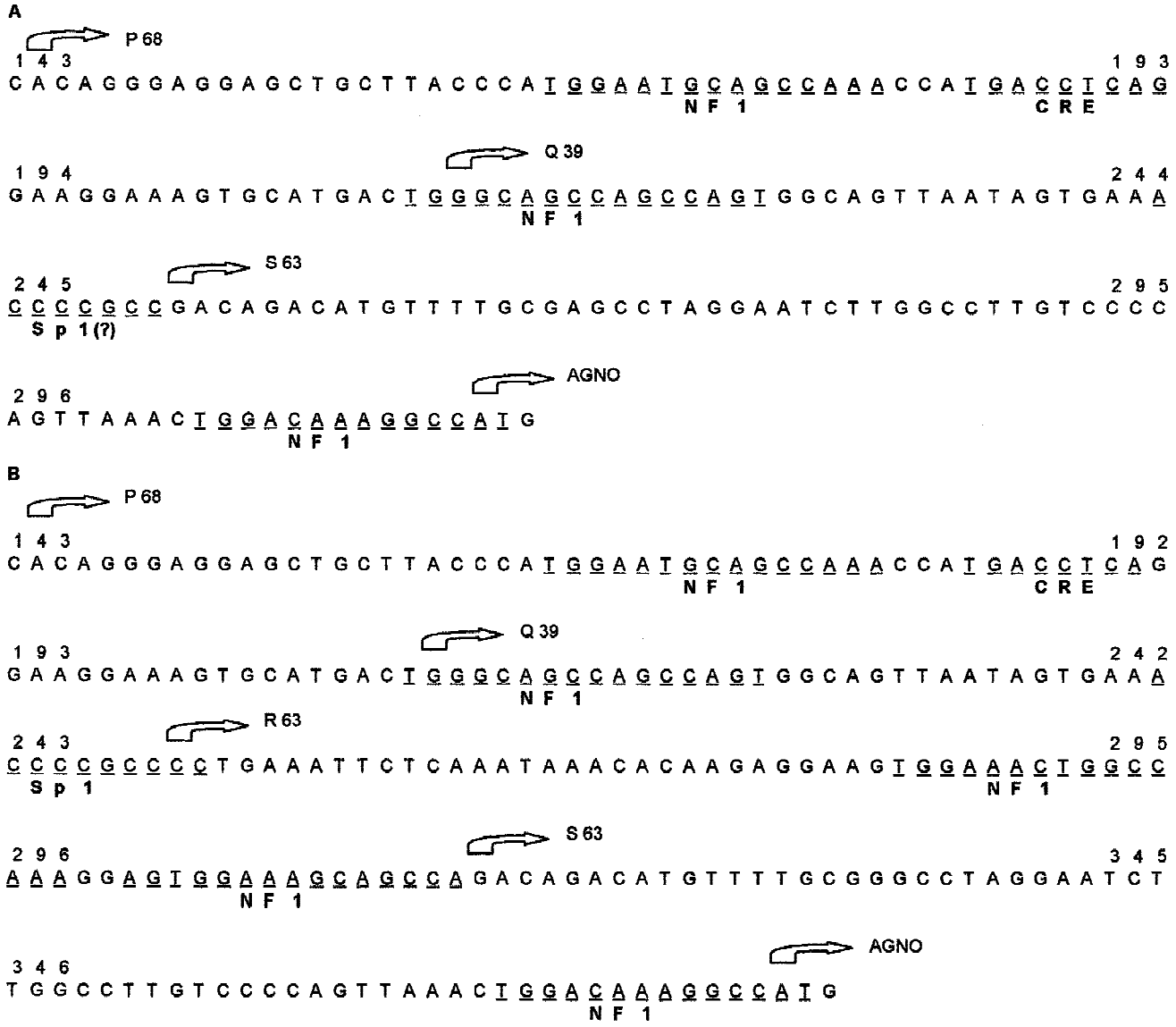

Fig. 3. Schematic diagram of nucleotide sequences of the non coding control region of BKV-DDP **(A)** and BKV-WW **(B)**. Blocks P, Q, R, and S are indicated and putative binding sites for transcription factors are overlined. The GenBank accession numbers of the sequences are respectively: U91605 and M15987.

nini et al., 1995]. In fact, the PQ strain was selected on passage of the Gardner strain (PPPQS) in the permissive Vero cell line [Ferguson and Subramani, 1994].

As far as the DDP strain isolated in PBMC is concerned, it should be pointed out that the NCCR organisation is similar to that of the PQ strain selected by Ferguson and Subramani after passages in Vero cells. Therefore the present study indicates that a similar structure of the NCCR can also be detected *in vivo* in the human host. Other DNA rearrangements of BKV NCCR may also occur *in vivo,* as observed in the TU strain, detected in the urine of immunosuppressed patients from Norway [Sundsfjord et al., 1990] and in the URO 1 strain, detected in tissue specimens from the urinary tract of Italian patients [Monini et al., 1995]. Another rearranged strain was found in Italy from several tumour tissue biopsies. DNA sequence analysis showed that viral isolates from all analysed samples had the same rearranged structure of the NCCR [Negrini et al., 1990].

On the basis of these and other studies [Watanabe et al., 1984; Grinell et al., 1988; Cassill and Subramani, 1989; Tavis et al., 1989], it has been suggested that rearrangements in the NCCR might affect tissue specificity, pathogenicity, and oncogenicity of polyomaviruses. In this context, it might be a possible explanation as to why BKV, which is assumed to remain latent in the kidney, can also be found in some tumours [Jin and Gibson, 1996].

As reported previously and confirmed further by this study, it is interesting to note that the analysis of the DNA organisation in urinary strains showed that all

viruses were characterised by a concomitant presence of the VP1 region and a NCCR, including the R_{63} box. By contrast, only 9/22 PBMC strains possessed the VP1 region and all of them presented the R_{63} box deletion. This finding suggests that the urinary strains (possessing both the VP1 and NCCR regions), associated with haemorrhagic cystitis or urethritis, are capable of efficient replication in epithelial cells or reactivation in the immunocompromised patients. It is possible that, through a viraemic phase, the virions infect the PBMCs. The fact that only a part of these PBMCs presents the structural VP1 gene, suggests two possibilities: 1) a different permissiveness of these cells for BKV or 2) different tropisms of BKV strains infecting the urinary epithelial cells or PBMC. The second alternative seems to be less probable, since the presence of VP1 is not constant in PBMC and could represent a step towards the complete loss of this region over time. One can speculate that the virus after the PBMC infection, loses the R_{63} box, by unknown mechanisms similar to those active when low permissive cells are infected *in vitro.* The deletion of this DNA segment, containing some crucial transcription binding sites, might determine a subsequent loss of the VP1 region. Obviously, due to the methodology employed, an integration of this region in a different DNA site, where the primers used are not able to detect it, cannot be ruled out. In fact, previous studies reported that BKV may be found both in episomal or integrated forms [Monini et al., 1995].

To support the possibility that the R63 box-deleted NCCR structure affects the efficiency of transcription and/or translation, the published list of binding sites for cellular factors was considered (Fig. 3). The box R deletion causes the loss of two nuclear-factor binding sites (namely, NF1) located in this block near the coding sequences for the late proteins. In addition, the Sp1 binding site sequence (located at the junction of boxes Q and R) appears shortened for the lack of the last two nucleotides. Other binding sites located in boxes P and Q are conserved, but the distance between these sites from the ATG codon for late transcription is modified (Fig. 3). NF1 is a major activator of both early and late BKV transcription *in vitro* [Cassill and Subramani, 1989; Chakraborty and Das, 1989, 1991] and can act as bidirectional transcriptional activator for BKV. The structure of this binding site is similar to the six copies of GC boxes found in the SV40 promoter. It is not yet known how different NF1 binding sites contribute to early and late transcription. All the NF1 binding sites in BKV have a twofold symmetry and are arranged in the same polarity in both the Gardner and Dunlop strains. This arrangement implies a cooperative interaction among them and bidirectionality in the NCCR [Chakraborty and Das, 1991]. Therefore, the loss of these two NF1 binding sites in the DDP strain could play an important role in the DNA transcription of the VP1 region together with the modified Sp-1 binding site, which can contribute to decrease the activity of the late promoter in B-lymphocytes, monocytes and macrophages, [Traavik et al., 1988; Johnsen et al., 1995].

In vitro experiments demonstrated that the PQ strain was more active transcriptionally in Vero cells than the WW strain and could replicate in and lyse these cells efficiently [Ferguson and Subramani, 1994]. The DDP variant here described, isolated in PBMC, seems to be less efficient for late transcription *in vivo* considering the absence of the VP1 in a high percentage of samples as a loss of this region or an integration within the host genome where the primers used cannot detect it. Other observations confirmed that a structure similar to that found in PBMC was also detectable in biopsies from different tissues (unpublished data). These results open the possibility that the rearrangements of BKV DNA in cells other than those of the urinary system may lead to cell transformation [De Mattei et al., 1995].

Some speculation may be raised regarding a lack of selective advantages for BKV-DDP strain taking into account the scarce information so far obtained. In fact, the Dunlop strain (lacking Q and R boxes) shows a weak late promoter/enhancer activity as compared with the TU strain [Moens et al., 1995]. In addition, the DeBruin (DB)d182 strain (without R-box) showed a higher transforming capacity on Rat2 cells than the variant containing the R-box [Tavis et al., 1990]. It may be that the non/low permissiveness of some types of cells may be mediated by structural DNA arrangements able to affect viral replication versus enhanced transformation ability. Further *in vivo* and *in vitro* studies are essential to clarify the finding presented in this communication.

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